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14. ABSTRACT

he inability to regrow functional limbs or limb segments lost to trauma or disease is a significant biomedical problem, with substantial associated monetary and quality-of-life implications for the nearly two million affected U. S. citizens and active service members. Development of in vivo therapies that restore regenerative capacity first requires an understanding of the basic gene regulatory networks controlling this biology. Thus, characterizing ancestral regulatory circuitry controlling regeneration is a necessary and direct route to identifying the mechanistic causes of regenerative failure in mammals. This proposal offers unique promise in guiding the targeted development of in vivo therapies to restore/augment human limb regeneration. We will leverage our recent discovery that regenerative ability is widespread in basal vertebrates to conduct the first comparative analysis of appendage regeneration that incorporates model systems from all major groups of limbed vertebrates- cartilaginous fishes, ray-finned fishes, and tetrapods. Our unique approach will identify novel functional requirements for genes/gene networks in regulating appendage regeneration by marrying a comparative organismal approach with state-of-the-art systems-level analyses of gene expression using next generation RNA sequencing and functional analysis of candidate regulators in the genetically tractable zebrafish model system through in vivo disruption of gene function.

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microRNAs	Zebrafish	Polypterus

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THE CENTER FOR REGENERATIVE BIOLOGY AND MEDICINE AT MOUNT DESERT ISLAND BIOLOGICAL LABORATORY

INTRODUCTION:

The inability to regrow functional limbs or limb segments lost to trauma or disease is a significant biomedical problem, with substantial associated monetary and quality-of-life implications for the nearly two million affected U. S. citizens and active service members. Development of *in vivo* therapies that restore regenerative capacity first requires an understanding of the basic gene regulatory networks controlling this biology. Thus, characterizing ancestral regulatory circuitry controlling regeneration is a necessary and direct route to identifying the mechanistic causes of regenerative failure in mammals. This proposal offers unique promise in guiding the targeted development of *in vivo* therapies to restore/augment human limb regeneration. We will leverage our recent discovery that regenerative ability is widespread in basal vertebrates to conduct the first comparative analysis of appendage regeneration that incorporates model systems from all major groups of limbed vertebrates- cartilaginous fishes, ray-finned fishes, and tetrapods. Our unique approach will identify novel functional requirements for genes/gene networks in regulating appendage regeneration by marrying a comparative organismal approach with state-of-the-art systems-level analyses of gene expression using next generation RNA sequencing and functional analysis of candidate regulators in the genetically tractable zebrafish model system through *in vivo* disruption of gene function.

BODY:

Specific Aim 1: Characterize mRNA expression profiles in regenerating salamander (*Ambystoma mexicanum*) limbs and *Polypterus senegalus* fins. This work will be accomplished by **a**) collecting tissue and mRNA from regenerating salamander and *Polypterus* appendages, **b**) characterizing gene expression profiles by DNA sequencing using Solexa/Illumina technology, **c**) assembling and annotating transcriptome sequence, **d**) identifying conserved regulators of limb/fin regeneration by quantitative bioinformatics analysis, and **e**) assessing functional requirements of candidate regulators.

Research Accomplishments: Work described for Specific Aim 1 was completed by the conclusion of Year 2 of the grant. Therefore, we have no new milestones to report for this Year 3 annual progress report.

Specific Aim 2: Characterize microRNA (miRNA) expression profiles in regenerating salamander (*Ambystoma mexicanum*) limbs and *Polypterus senegalus* fins. This work will be accomplished by **a**) collecting tissues and isolating small RNA from regenerating salamander and *Polypterus* appendages, **b**) characterizing miRNA expression profiles generated by DNA sequencing using Solexa/Illumina technology, **c**) annotating miRNAs and characterizing their expression profiles in limb/fin regeneration, **d**) predicting miRNA targets and correlating miRNA expression with predicted targets, and **e**) assessing functional roles of candidate miRNAs.

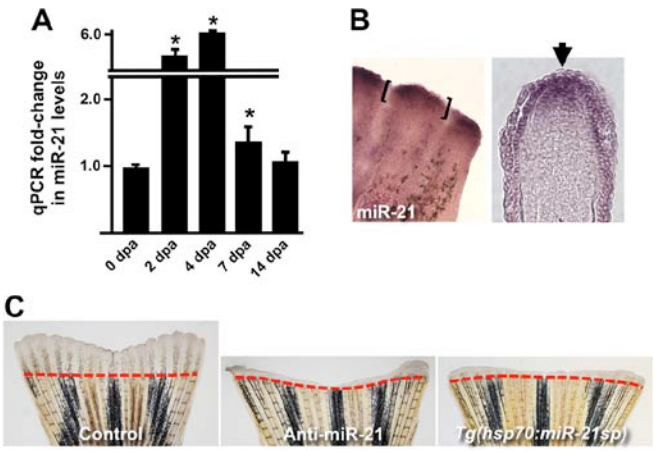

Figure 1. miR-21 is required for blastema formation. A) Real-time qPCR studies reveal the temporal expression profile of miR-21 during appendage regeneration. **B)** *In situ* hybridization studies to detect miR-21 expression. Brackets and arrowhead mark miR-21 expression. **C)** miR-21 depletion with antisense oligonucleotides or with *Tg(hsp70:miR-21^{sp})* strain inhibits blastema formation. (Red dashed line = original amputation plane).

Research Accomplishments: Regeneration of complex appendage tissue requires differentiated, quiescent tissues being transformed into proliferative, progenitor cells that accumulate to form the blastema[7]. This naturally reprogrammed, indispensable progenitor tissue is the source of all regenerating appendage tissue, including connective tissue, nerves, blood vessels, pigment cells and epidermis. Blastema formation requires the rapid modulation of genetic programs.

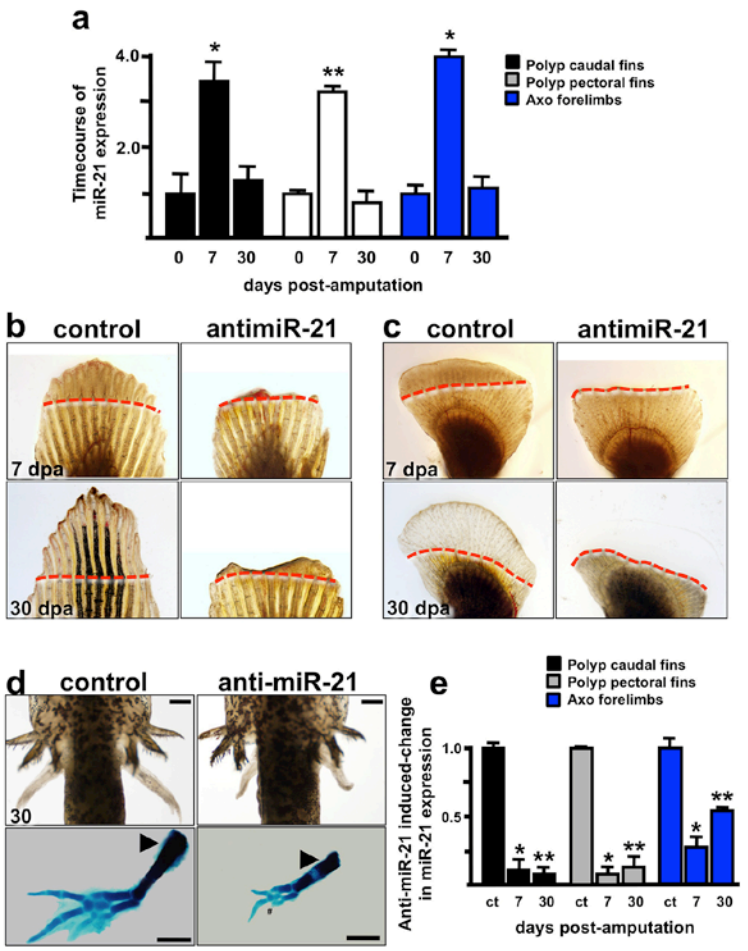

Figure 2. miR-21 is required for axolotl and Polypterus limb regeneration. **A)** Real-time qPCR studies reveal the temporal expression profile of miR-21 during appendage regeneration. **B-C)** *Polypterus* caudal fin (B) and pectoral fin (C) regeneration in control and antimiR-21 treated animals. (Red dashed line = original amputation plane). **D)** Limb regeneration in axolotl treated animals. (Arrowhead = original amputation plane). E) Real-time qPCR studies showing changes in miR-21 levels after LNA-antimiR-21 treatment.

Gene regulatory networks are controlled at multiple levels. miRNAs are key regulatory factors during gene expression with the unique ability to modulate hundreds of target genes, thus making them ideal candidates to control the cellular process of complex tissue regeneration. Previously, we isolated total RNA, enriched for small microRNAs (less than 200 nucleotides) and performed deep sequencing for small RNAs. Our analyses identified 8 different miRNAs, including miR-21, miR-181a, miR-7a, let-7j, miR-130c, miR-338, miR-204 and miR-2184 that are shared between regenerating axolotl forelimbs, zebrafish caudal fins and *Polypterus* pectoral fins. miR-21 was the most highly upregulated miRNA in all three model systems examined. In the absence of miR-21 activity, appendage regeneration was defective in both zebrafish and *Polypterus* fin appendages and axolotl limbs (Fig. 1, 2). *Polypterus* caudal and pectoral fins exhibited no regeneration due to failure in blastema formation. In axolotls however, the primary role for miR-21 appears to be pattern formation. miR-21 depleted animals regenerate shorter and thicker limbs when compared to control animals (Fig. 2). In short, our studies indicate that miR-21 is an essential, conserved component of the regenerative genetic circuit.

To define the potential mechanism of action for miR-21 function in response to appendage injury, we first used real-time qPCR studies to identify changes in genetic markers of regeneration in the zebrafish caudal fins. Consistent with a strong defect in regenerative outgrowth, the blastema marker *msxb*, the cell cycle regulator *mps1* and the ETS transcription factor *pea3* were all downregulated when miR-21 activity was depleted (Fig. 3). Interestingly, key components of the Fibroblast growth factors (Fgf) were elevated in expression despite the strong block in regeneration. We were surprised to observe significantly elevated levels of *fgf20a* and *mkp3* given that increased expression is associated with normal regenerative outgrowth of appendage tissue (Fig. 3A). How can this paradoxical result between gene expression of regeneration markers and the lack of regeneration be explained?

One possible explanation is that miR-21 fine-tunes Fgf expression levels, keeping its activity within a permissive window that promotes cellular dedifferentiation (Fig. 3C). Appendage regeneration and limb development are particularly sensitive to Fgf expression levels. For instance, previous zebrafish appendage regeneration studies showed that mutations in *fgf20a* inhibits blastema formation and regenerative outgrowth. Likewise, inhibition of the entire Fgf signaling network with activation of the *Tg(hsp70:DN-Fgfr1)* transgene culminated in regeneration inhibition. Conversely, high Fgf levels during development is inhibitory, functioning to terminate limb bud outgrowth. Since, *fgf20a* has a predicted binding site for miR-21 in the 3' UTR, we propose that miR-21 is a critical regulator of Fgf activity during tissue repair and regeneration. Upon appendage injury, the normal upregulation of miR-21 dampens the sudden increase in Fgf activity, thus positioning Fgf expression within a conducive window for tissue repair and regeneration (Fig. 3C).

Growth factor sequester model: In this alternative sequester model, we hypothesize that growth factors like *fgf20a* are being activated normally in response to injury but are sequestered due to inhibition of extracellular matrix (ECM) remodeling by decreases in matrix metalloproteinase (MMPs) activity (Fig. 3D). MMPs are normally held in an inactive state through their association with tissue inhibitors of MMPs (timps). Disrupting the MMP/timp balance is critical for remodeling of ECM space in order to promote cellular migration and proliferation and cell-cell communication during development.

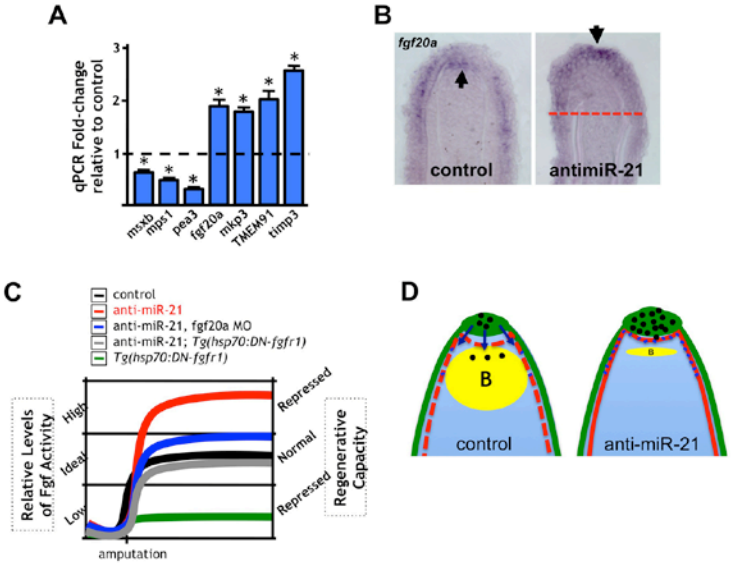

Figure 3. Two models of miR-21 mechanism of action. A) Real-time qPCR studies show decreases in *msxb*, *mps1* and *pea3* and elevation of *fgf20a*, *mkp3*, *TMEM91* and *timp3* expression levels. **B)** *In situ* hybridizations of control and antimiR-21 fin sections to detect *fgf20a* expression. Arrowhead represent *fgf20a* expression and red dashed line = original amputation plane. **C)** A depiction of the Growth factor threshold model. Fgf activity must be tightly controlled and maintained in an "ideal" range for regeneration to proceed normally. Low or high levels of Fgf represses regeneration. **D)** An illustration of the Growth factor sequester model. Fgf20a (black circles) migration is essential to activate blastema (yellow circle with B) formation. Fgf20a ligand passage to the mesenchyme requires changes to cellular adhesion and ECM remodeling of the basal-epidermis tissue (red line). These remodeling events of the basal-epidermis are inhibited under conditions of miR-21 depletion. (* = Student's ttest p-value <0.05).

We predict that under normal regeneration conditions, *fgf20a* activation is localized to the wound epidermis and then disperses into neighboring proximal cells whereby it induces cellular dedifferentiation and subsequent blastema formation. However, migration of *fgf20a* requires remodeling of the basal epidermal tissue, which lies between the wound epidermis and the mesenchyme. As cellular dedifferentiation progresses, levels of *fgf20a* are diminished through interactions with miR-21. In *Tg(hsp70:miR-21^{sp})* or antimiR-21 treated animals, *in situ* hybridizations studies demonstrate that indeed *fgf20a* expression is confined to the thickened epidermis (Fig. 3B). Furthermore, two validated miR-21 target genes, tissue inhibitor of metalloproteinase 3 (*timp3*) and ECM transmembrane factor factor TMEM91, are highly enriched in appendage tissues devoid of miR-21 activity (Fig. 3A). In addition, preliminary studies with a pan-cadherin antibody to detect intercellular adhesion suggest that animals lacking miR-21 activity have tighter cellular association and are devoid of changes in cellular morphology within the basal-epidermal tissue layer. Collectively, we believe these results suggest that miR-21 may promote dispersal of growth factor ligands via remodeling of the ECM of the basal-epidermis tissue. We are working to test these two models of miR-21 mechanism of action with various cellular and molecular approaches.

Specific Aim 3: Determine a genetic positional memory code for appendage regeneration. This work will be accomplished by **a**) isolating tissue and RNA from uninjured and regenerating zebrafish caudal fins, **b**) profiling miRNAs with microarray hybridization technology and **c**) filter and categorize the dataset into increasing and decreasing miRNA expression.

Research Accomplishments: Work described for Specific Aim 3 was completed by the conclusion of Year 2 of the grant. Therefore, we have no new milestones to report for this Year 3 annual progress report.

Specific Aim 4: Define requirements for region-specific regulatory factors in maintaining positional memory. This work will be accomplished by **a**) validating miRNA expression using Northern blot hybridization and/or real-time quantitative PCR, **b**) determining spatial resolution of miRNAs during regenerative states and **c**) performing functional studies on miRNAs using antisense oligonucleotides to determine the effects on regeneration.

Research Accomplishments: In our functional studies we noted that animals with mild miR-21 inhibition regenerated appendage tissues with altered patterning. This defect in fin bone bifurcation patterning phenocopy appendages with exogenous RA treatment (Fig. 4A). Given these similarities, we hypothesized

that miR-21 may control RA activity during regenerative tissue patterning. First, we asked if miR-21 and RA expression is regionalized during appendage regeneration. We divided the caudal fin into three zones along the proximal-distal axis, termed PD1-3, to represent defined areas of the caudal fin. We used a razor blade to remove 75%, 50% or 25% of the caudal fin (Fig. 4B). We believe incorporating three different amputation planes enhances the probability of identifying genetic factors that are expressed in a gradient than previous work that relied solely on two planes of injury. For intact appendage samples, we collected tissues 2-bony segments proximal to this initial amputation plane. In studies of regeneration, we collected the regenerated tissues at 4 dpa because it represents a stage in appendage regeneration when positional cues have been determined. RNA was isolated from uninjured and regenerating tissues using Tri-reagent in accordance to the manufacturer's protocol and used for real-time qPCR studies (Sigma). Under conditions of no injury and during tissue regeneration, our studies showed that miR-21 expression is inversely expressed with components of the RA pathway, including *raldh2*, *RXR-α* and *RXR-Δ*. Whereas miR-21 expression is low at PD1, components of the RA pathway show elevated expression levels (Fig. 4C, D). Most importantly, the dataset suggests that positional identity is maintained under conditions of homeostasis and re-established in freshly injured appendage tissue and miR-21 may be a critical component of this regulatory network.

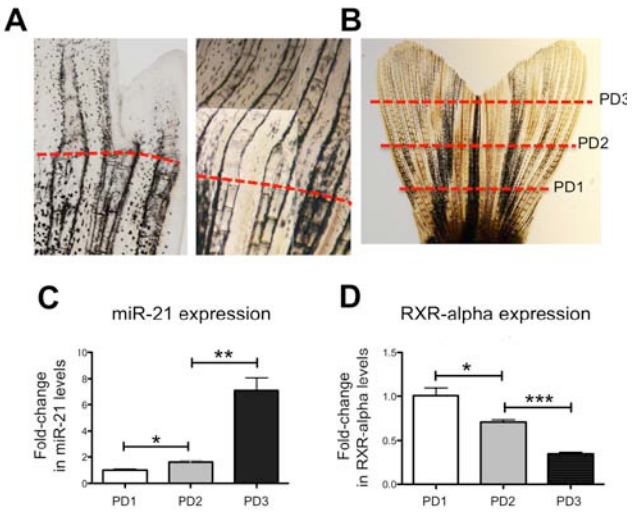

Figure 4. Loss of miR-21 phenocopy retinoic acid activity overexpression. A) Regenerating caudal fins treated with antimiR-21 depletion (Left) or retinoic acid treatment[1] (Right). **B)** Proximal-distal amputation planes in the caudal fin. **C-D)** Real-time qPCR studies show the regionalized expression of miR-21 or *RXR-alpha* during caudal fin regeneration. (*,**, *** = Student's ttest p-value <0.01, 0.005, 0.001).

Specific Aim 5: Grow Mount Desert Island Biological Laboratory as a collaborative center for the Regenerative Biology and Medicine research community. This work will be accomplished by establishing a MDIBL Visiting Scholars Program in Regenerative Biology.

Research Accomplishments: We have reviewed visiting scientist applications and have awarded fellowships for 2014. All awards were made to applications focused on deciphering cellular processes during development and regulation of stress biology. Recent studies have shown that these cellular processes are key to understanding how the regenerative genetic circuit is activated. Below are the scientists that were awarded fellowships this year.

1. Ken Poss, Ph.D., Duke University Medical Center: Heart regeneration in the zebrafish.
2. Larissa Williams, Ph.D., Bates College: The importance of transcription factors in the Nfe2 family in the embryonic response to environmental toxicant exposure.
3. Zoya Ignatova, Ph.D., University of Potsdam, Germany: The effect of intrinsic (repetitive mutations and aging) and extrinsic (environmental stress) stress on translation dynamics.
4. Jorge Contreras, Ph.D., New Jersey Medical School: The role of Connexin channels in development and normal organ function.

KEY RESEARCH ACCOMPLISHMENTS:

- Identified miR-21 as the most highly upregulated miRNA in Axolotl, *Polypterus* and zebrafish limb injuries.
- Initiated functional studies to examine the roles of miR-21 during cellular reprogramming in response to injury and regeneration.
- Identified a miR-21-retinoic acid genetic axis for positional memory and tissue patterning during zebrafish appendage regeneration.
- Successfully recruited and awarded regeneration fellowships to prominent regeneration scientists.

REPORTABLE OUTCOMES:

40th Maine Biological and Medical Sciences Symposium (abstract):

MicroRNA Control of Appendage Regeneration

King, BL and Yin, VP
[1]Mount Desert Island Biological Laboratory, Salisbury Cove, Maine 04672 USA

The long-range goal of this proposed research is to dissect the molecular regulation of vertebrate limb regeneration, and apply this information toward designing therapies that restore regenerative responses in humans. The inability to regrow functional limbs or limb segments lost to trauma or disease is a significant biomedical problem, with substantial associated monetary and quality-of-life implications for the nearly two million US citizens. Development of therapies to restore regenerative capacity first requires an understanding of the basic gene regulatory networks controlling this biology. While this capacity is limited only to the very distal tips of digits in mammals, adult teolost fish and urodele amphibians have championed regeneration of entire appendages, replacing bone, connective tissue, epidermis, nerves, blood vessels and pigment cells. The key feature that underscores appendage regeneration is the formation of the blastema, a highly proliferative progenitor tissue that arises through dedifferentiation of existing cells. Our goal is to identify a core genetic signature that regulates the formation and maintenance of the blastema by comparing high-throughput RNA sequencing (RNA-Seq) datasets from regenerating axolotl forelimbs, bichir pectoral fins and zebrafish caudal fins.

Initiation and progression of appendage regeneration involves modulating multiple genetic programs through regulatory factors. MicroRNAs (miRNAs) are short highly conserved non-coding genes that suppress expression of target genes and thereby control multiple genetic programs. Given the important regulatory roles of miRNAs, and evolutionary conservation, we hypothesize that differentially expressed miRNAs define a conserved genetic regulatory circuit important for appendage regeneration. We found six upregulated and six down-regulated miRNAs common to all three model systems. The most highly up-regulated miRNA in the three models was miR-21. We are currently analyzing corresponding mRNA-Seq data to find candidate target genes for miR-21 and the other commonly expressed miRNAs. One promising candidate gene is the matrix metalloproteinase inhibitor, *reck*, that is commonly down-regulated in axolotl and bichir and a known miR-21 target gene in human glioblastoma cell lines.

5th Biennial National IDeA Symposium of Biomedical Research Excellence (Abstract):

MicroRNA Control of Appendage Regeneration

Benjamin L. King, Heather R. Carlisle and Viravuth P. Yin
Mount Desert Island Biological Laboratory, Salisbury Cove, ME

Background and Objective: Our long-range goal is to dissect the molecular regulation of vertebrate limb regeneration, and apply this information toward designing therapies that restore regenerative responses in humans. Development of therapies to restore regenerative capacity first requires an understanding of gene regulatory networks controlling this biology. While this capacity is limited only to distal tips of digits in mammals, adult teleost fish and urodele amphibians have championed regeneration of entire appendages. The key feature of appendage regeneration is the formation of the blastema, a highly proliferative progenitor tissue that arises through dedifferentiation of existing cells. Our goal is to identify a core genetic signature that regulates the formation and maintenance of the blastema by comparing gene expression profiles in regenerating axolotl forelimbs, bichir pectoral fins and zebrafish caudal fins. Given the important regulatory roles of microRNAs and evolutionary conservation, we hypothesize that microRNAs define a conserved genetic regulatory circuit important for appendage regeneration.

Methods: Illumina RNA sequencing of small RNAs and mRNAs, qPCR validation and anti-miR knockdown.

Results: We found six up-regulated and six down-regulated microRNAs common to all three model systems. MiR-21 was consistently up-regulated and anti-miR-21 knockdown inhibited regeneration in each model.

Discussion and Conclusions: MicroRNAs, such as miR-21, are required for appendage regeneration and appear to constitute a conserved regulatory circuit for regeneration.

11th International Conference on Zebrafish Development and Genetics (abstract):

Regulation of Zebrafish Fin Regeneration by miR-21

Heather Carlisle, Ashley Smith, Benjamin King and Viravuth P. Yin

Davis Center for Regenerative Biology and Medicine, Mount Desert Island Biological Labs, Salisbury Cove, ME 04672, USA

Appendage regeneration is defined by the transformation of quiescent, differentiated tissues into highly proliferative and regenerative blastemal cells. These dramatic cellular changes are accompanied with rapid modulation of gene expression, thus implicating miRNAs. Here we performed deep-sequencing studies to identify shared regeneration miRNAs among zebrafish caudal fins. Real-time qPCR analysis confirmed miR-21 is one of the most highly upregulated miRNAs in response to injury. Subsequent qPCR analysis shows this upregulation in all five zebrafish fin types. *In situ* hybridization studies in zebrafish caudal fins reveal miR-21 expression is localized to the basal-epithelial tissue layer and distal blastemal cells. Experimental depletion of miR-21 levels with antisense oligonucleotides culminated in regenerative outgrowth and patterning defects in all fin types. Furthermore, we show in the zebrafish that miR-21 is essential to activate blastema formation and cell proliferation and depletion of this microRNA effects multiple signaling pathways. Using an integrated bioinformatics approach, we have identified *fgf20A, bmp3, and timp3* as miR-21 putative target genes. Collectively, our studies implicate miR-21 as a key component of a miRNA genetic circuit for repair and regeneration of complex appendage tissues.

11th International Conference on Zebrafish Development and Genetics (abstract):

Genetic determinants of positional memory during appendage regeneration

FitzSimons, ML[1,2], Carlisle, HR[2], Tian, C[1,2], and Yin, VP[1,2]
[1]GSBSE, University of Maine, Orono, ME 04469
[2]Mount Desert Island Biological Laboratory, Salisbury Cove, ME 04672

In the United States alone, almost two million people live with limb loss, primarily the result of dysvascular pathology or trauma associated amputations. Amputees suffer from significantly lowered health status, as well as increased rates of mortality; therefore, it is critical to elucidate mechanisms that promote successful limb regeneration. While humans possess limited regenerative capacity, the zebrafish displays a remarkable ability to completely regenerate damaged or lost appendages; however the mechanisms governing this process are not completely understood. During appendage regeneration, an information gradient along appendage axes is believed to regulate positional memory – the meticulous and appropriate replacement of complex tissues. Because both microRNAs (miRNAs) and retinoic acid (RA) are recognized to regulate appendage regeneration, we examined the gradient expression of miRNAs and components of the RA signaling pathway in tissues isolated from three planes along the proximal-distal (P/D) axis in the zebrafish caudal fin. Initial microarray analysis identified miRNAs with significant changes in expression during regeneration. Among these, miR-21 revealed the most dramatic upregulation. qPCR studies demonstrated that expression of miR-21 increases in a gradient along the P/D axis in both uninjured and regenerate fins. In contrast, two components of the RA signaling pathway, RALDH-2 and RXR-αa, are downregulated along the same gradient. The inverse correlation between expression of miR-21 and RA pathway members suggests that miR-21 may play a role in modulating RA signaling. This hypothesis is supported by previous experiments, which demonstrated that the RA co-receptor RXR-Δ is a direct target of miR-21, and that zebrafish caudal fins treated with anti-miR-21 are characterized by proximalization of structures during regeneration, reflecting patterns observed with RA treatment. Our results suggest that miR-21 regulates positional memory through targeting key components of the RA signaling pathway. This study presents important preliminary data contributing to our understanding of successful limb regeneration.

CONCLUSIONS:

In order to develop potential therapies to restore and/or augment human limb regeneration, we must first understand the molecular regulation of appendage regeneration in vertebrates that have retained enhanced regenerative capacity during evolution. Two critical limitations have impeded progress in this area: 1) lack of diverse experimental animals has precluded the powerful comparative approaches that have vertically advanced other fields of regenerative medicine such as stem cell biology; 2) unbiased functional genomic approaches have not been fully exploited. The experimental design we advance in this proposal integrates two innovative approaches to address these issues, and distinguishes this work as a unique and complementary extension of current projects in TATRC's portfolio. We have and will continue to use a novel comparative approach employing phylogenetically diverse experimental organisms, and adopt unbiased systems-level approaches to identify and dissect the gene networks initiating and maintaining regenerative responses in vertebrate limbs.

In brief, a major finding during Year 3 has been the dual role for miR-21 during limb/appendage regeneration. In the early phases of injury response, increases in miR-21 levels are required to induce cellular

dedifferentiation and proliferation of the regenerative blastema. Interestingly, following blastema formation, miR-21 expression is regionalized along the regeneration axis. Disruption of this proximal-distal gradient alters tissue patterning. It is our expectation that our recent progress and the collective aims of this proposal will provide novel fundamental insight into the molecular control of appendage regeneration, vertically advancing our understanding of the mechanistic causes of regenerative failure in mammals, and ultimately guiding the targeted development of *in vivo* therapies to restore/augment human limb regeneration.

REFERENCES: N/A

APPENDICES: N/A

SUPPORTING DATA: N/A